UNIVERSITI PUTRA MALAYSIA

INDUCTION OF APOPTOSIS BY 2',3'-EPOXYISOCAPNOLACTONE AND 8-HYDROXYISOCAPNOLACTONE-2',3'- DIOL ISOLATED FROM MICROMELUM MINUTUM IN HUMAN T-LYMPHOCYTE LEUKEMIA CEM-SS CELLS

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TAN BOON KEAT

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By

TAN BOON KEAT

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirement for the Degree of Master of Science

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

INDUCTION OF APOPTOSIS BY 2',3'-EPOXYISOCAPNOLACTONE AND 8-HYDROXYISOCAPNOLACTONE-2',3'-DIOL ISOLATED FROM *MIRCOMELUM MINUTUM* IN HUMAN T-LYMPHOCYTE LEUKEMIA CEM-SS CELLS

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March 2006

Chairman : Professor Abdul Manaf Ali, PhD

Faculty : Institute of Bioscience

2',3'-epoxyisocapnolactone and 8-hydroxyisocapnolactone-2',3'-diol are two bioactive compounds isolated from the leaves of *Micromelum minutum*. The cytotoxic effect of the compounds was tested on a variety of human cell lines respectively using MTT assay. They were found to be most sensitive against human T-lymphoblastic leukemia cells (CEM-SS). The inhibition effect of 2',3'-epoxyisocapnolactone and 8-hydroxyisocapnolactone-2',3'-diol at 50% of cell population (IC_{50}) was found to be 4.6 μg/ml (13.5 μM) and 3 μg/ml (7.8 μM) on CEM-SS cells, respectively. Besides that, the inhibitor effect of the compounds on other human cells were found to be 13.4 μg/ml (39.2 μM) and 9.0 μg/ml (23.9 μM) on cervical carcinoma cells (HeLa), 14.2 μg/ml (41.5 μM) and 7.7 μg/ml (20.5 μM) on colon adenocarcinoma cells (HT29), 7.4 μg/ml (21.6 μM) and 5.9 μg/ml (15.7 μM) on hepatocarcinoma cells (HepG2), 6.5 μg/ml (19.0 μM) and 7.1 μg/ml (18.9 μM) on transform liver cells (Chang). For comparative purposes, the IC_{50}

of several clinical cytotoxic drugs against CEM-SS cells were determined. The inhibitor effect of the compounds were more significant compared with methotrexate [IC_{50} = >30 μg/ml (66.1 μM)], cytosine arabinoside [IC_{50} = >30 μg/ml (123.5 μM)] and colchicines [IC_{50} = 8 μg/ml (20.1 μM)]. The compounds also shown near similar IC_{50} concentration as compare with cis-diamine dichloroplatinum [IC_{50} = 3 μg/ml (10.1 μM)], vinorelbine [IC_{50} = 3 μg/ml (3.9 μM)] and doxorubicin [IC_{50} = 2.4 μg/ml (4.1 μM)]. Furthermore, from proliferation assay study, the compounds were significantly inhibiting the proliferation of cells at IC_{50} value. From the morphological observation and agarose gel electrophoresis, apoptosis of the compounds on CEM-SS cells was determined. By using phase contrast, fluorescence and electron microcopies, observation on morphological alterations indicating apoptosis was evaluated. From DNA fragmentation, Acridine orange and Propidium iodide staining and DNA content analyses, the compounds were confirmed to have ability in promoting apoptosis. However, the percentage of apoptosis induced is low and the event is time-dependent. At high concentration of 10 μg/m, 2',3'-epoxyisocapnolactone and 8-hydroxyisocapnolactone-2',3'-diol induced necrosis. Furthermore, 8-hydroxyisocapnolactone-2',3'-diol also exhibited better cytotoxicity compared to 2',3'-epoxyisocapnolactone. The induction time for apoptosis by 8-hydroxyisocapnolactone-2',3'-diol in CEM-SS is earlier than 2',3'-epoxyisocapnolactone, which is 4 hours and 12 hours after treatment. Based on the results obtained, 2',3'-epoxyisocapnolactone and 8-hydroxyisocapnolactone-2',3'-diol are able to induced apoptosis.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

KESAN INDUKSI APOPTOSIS OLEH 2',3'-EPOXYISOCAPNOLACTONE DAN 8-HYDROXYISOCAPNOLACTONE-2',3'-DIOL YANG DIASINGKAN DARI *MICROMELUM MINUTUM* KE ATAS JUJUKAN SEL CEM-SS T-LYMPHOCYTE LEUKEMIK MANUSIA

Oleh

TAN BOON KEAT

Mac 2006

Pengerusi : Profesor Abdul Manaf Ali, PhD

Fakulti : Institusi Biosains

2',3'-epoxyisocapnolactone dan 8-hydroxyisocapnolactone-2',3'-diol merupakan dua jenis sebatian yang diasingkan dari daun *Micromelum minutum*. Kesan sitotoksik oleh kedua-dua sebatian ke atas pertumbuhan perbagai jenis jujukan kanser sel manusia telah diuji dengan teknik MTT. Mereka didapati lebih sensitive ke atas jujukan sel T-lymphoblastik leukemik manusia (CEM-SS). Kesan perencatan oleh 2',3'-epoxyisocapnolactone and 8-hydroxyisocapnolactone-2',3'-diol pada 50% dari sel populasi (IC_{50}) didapati sebanyak 4.6 μg/ml (13.5 μM) dan 3 μg/ml (7.8 μM) masing-masing ke atas sel CEM-SS. Selain itu, kesan perencatan oleh kedua-dua sebatian aktif di atas jujukan sel manusia yang lain juga didapati sebanyak 13.4 μg/ml (39.2 μM) dan 9.0 μg/ml (23.9 μM) ke atas sel servikal karsinoma (HeLa), 14.2 μg/ml (41.5 μM) dan 7.7 μg/ml (20.5 μM) ke atas sel adenokarsinoma usus (HT29), 7.4 μg/ml (21.6 μM) dan 5.9 μg/ml (15.7 μM) ke atas sel hepatokarsinoma (HepG2), 6.5 μg/ml (19.0 μM) dan 7.1

μg/ml (18.9 μM) ke atas sel hati yang normal (Chang). Sebagai bandingan, IC_{50} dari beberapa jenis ubat pasaran ke atas sel CEM-SS juga diujikan. Kesan perencatan oleh kedua-dua sebatian semulajadi adalah lebih berkesan berbanding dengan ubat-ubatan pasaran seperti methotrexate [IC_{50} = >30 μg/ml (66.1 μM)], cytosine arabinoside [IC_{50} = >30 μg/ml (123.5 μM)] dan colchicines [IC_{50} = 8 μg/ml (20.1 μM)]. Kedua-dua sebatian juga menunjukan aktiviti IC_{50} yang agak sama berbanding dengan cis-diamine dichloroplatinum [IC_{50} = 3 μg/ml (10.1 μM)], vinorelbine [IC_{50} = 3 μg/ml (3.9 μM)] dan doxorubicin [IC_{50} = 2.4 μg/ml (4.1 μM)]. Selain itu, dari pengajian atas teknik pertumbuhan, kedua-dua sebatian adalah ketara dalam perencatan pertumbuhan sel pada IC_{50}. Kesan apoptosis bagi kedua-dua sebatian aktif ke atas sel CEM-SS telah diperolehi daripada pemerhatian morfologi dan elektrophoresis gel agaros. Dengan mengunakan teknik mikroskop fasa perbezaan, fluoresent dan elektron, pemerhatian ke atas perbezaan morfologi yang berkaitan dengan apoptosis telah dijalankan. Dari keputusan DNA fragmentasi, pewarnaan AO/PI dan pengajian kandungan DNA, kedua-dua sebatian aktif telah menunjukan kebolehannya dalam menrangsangkan induksi apoptosis. Akan tetapi, peratus bagi induksi apoptosis adalah rendah and kemunculan kesan apoptosis adalah bergantung pada masa rawatan. Bila 2',3'-epoxyisocapnolactone and 8-hydroxyisocapnolactone-2',3'-diol dirawat dengan dos yang tinggi (10 μg/ml), ia akan menrangsangkan nekrosis. Selebih daripada itu, 8-hydroxyisocapnolactone-2',3'-diol juga menunjukkan aktiviti sitotoksik yang lebih baik berbanding dengan 2',3'-epoxyisocapnolactone. Kemunculan kesan apoptosis di dalam 8-hydroxyisocapnolactone-2',3'-diol adalah lebih awal berbanding dengan 2',3'-epoxyisocapnolactone, iaitu 4 jam and 12 jam selepas rawatan. Berdasarkan keputusan yang diperolehi, kedua-dua sebatian

semulajadi (2',3'-epoxyisocapnolactone and 8-hydroxyisocapnolactone-2',3'-diol) boleh disimpulkan sebagai agen sitotoksik potensi yang boleh merangsangkan apoptosis.

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I certify that an Examination Committee has met on 20th March 2006 to conduct the final examination of Tan Boon Keat on his Master of Science thesis entitled "Induction of Apoptosis by 2',3'-Epoxyisocapnolactone and 8-Hydroxyisocapnolactone-2',3'-diol Isolated from *Micromelum minutum* in Human T-Lymphocyte Leukemia CEM-SS Cells" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

Fauziah Othman, PhD
Associate Professor,
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Chairman)

Johnson Stanslas, PhD
Lecturer,
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Internal Examiner)

Muhajir Hamid, PhD
Lecturer,
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Internal Examiner)

Shaida Fariza Sulaiman, PhD
Associate Professor
School of Biological Sciences
Universiti Sains Malaysia
(External Examiner)

HASANAH MOHD. GHAZALI, PhD
Professor/Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:

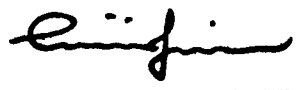
26 APR 2006

This thesis submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirements for the degree of Master of Science. The members of the Supervisory Committee are as follows:

ABDUL MANAF ALI, PhD
Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Chairman)

MAWARDI RAHMANI, PhD
Professor
Faculty of Sciences
Universiti Putra Malaysia
(Member)

NOORJAHAN ALITHEEN, PhD
Lecturer
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Member)



AINI IDERIS, PhD
Professor/Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 11 MAY 2006

DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations, which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

TAN BOON KEAT

Date: 24 / 4/06

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LIST OF ABBREVIATIONS

%	Percentage
ALL	Acute Lymphocytic Leukemia
ANLL	Acute Non-Lymphocytic Leukemia
AO	Acridine Orange
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
bp	Base pair
CEM-SS	T-Lymphoblastic Leukemia
CGM	Complete Growth Medium
Chang	Normal Liver Cell
CO_2	Carbon dioxide
Da	Dalton
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid Di-Sodium Salt
ELISA	Enzyme linked immunosorbent assay
FBS	Fetal Bovine Serum
HeLa	Cervical Carcinoma
HepG2	Hepato Carcinoma
HT29	Colon Carcinoma
IC_{50}	Inhibition Concentration 50%
mg	Milligram
ml	Milliliter

MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
nm	Nanometer
O.D.	Optical Density
PBS	Phosphate Buffered Saline
PI	Propidium Iodide
RNA	Ribonucleic acid
RPM	Rotation per Minute
SDS	Sodium dodecyl sulfate
SEM	Scanning Electron Microscope
UV	Ultra violet
µg	Microgram
µl	Microliter

CHAPTER 1

INTRODUCTION

Cancer chemotherapy, the treatment or control of cancer using anticancer drugs which are highly toxic medications that destroy cancer cells by interfering with their growth or preventing their reproduction (Altman and Sarg, 1992). It has played a role in cancer treatment for almost half a century. Years of testing and research have proved chemotherapy to be an effective cancer treatment. It may be the only treatment, or it may be used in combination with other treatments, including surgery and radiation therapy. Chemotherapy works by killing rapidly dividing cells. These cells include cancer cells, which continuously divide to produce more cells, and healthy cells that divide quickly, such as those in bone marrow, gastrointestinal tract, reproductive system and hair follicles. Healthy cells usually recover shortly after chemotherapy is complete (Mayo Foundation for Medical Education and Research, 2003). It differs from surgery or radiation in that it is always used as a systemic treatment. In chemotherapy the medicines travel throughout the whole body or system rather than being confined or localized to one area such as the breast, lung, or colon. Thus chemotherapy can reach cancer cells that may have spread to other parts of the body.

More than 100 drugs are currently used for chemotherapy, either alone or in combination. Many more are expected to become available. These chemotherapy medicines are vary widely in their chemical composition, how they are taken, their usefulness in treating

specific forms of cancer, and their side effects. New medications are first developed through laboratory research in test tubes and animals. Then, their safety and effectiveness are tested for clinical trials in humans (American Cancer Society, 2001).

Chemotherapy drugs are divided into several categories based on how they affect specific chemical substances within cancer cells, which cellular activities or processes the drug interferes with, and which specific phases of the cell cycle the drug affects. Most chemotherapy is given as a combination of drugs that work together to kill cancer cells. Some of the types of chemotherapy medications commonly used to treat cancer include: 1) Alkylating agents. These medications interfere with the growth of cancer cells by blocking the replication of DNA. Examples of alkylating agents include busulfan, cisplatin, carboplatin, chlorambucil, cyclophosphamide, ifosfamide, dacarbazine (DTIC), mechlorethamine (nitrogen mustard), and melphalan. 2) Antimetabolites. These drugs block the enzymes needed by cancer cells to live and grow. Examples of antimetabolites include 5-fluorouracil, capecitabine, methotrexate, gemcitabine, cytarabine (ara-C), and fludarabine. 3) Antitumor antibiotics. These antibiotics are different from those used to treat bacterial infections. It interferes with DNA, blocking certain enzymes and cell division, and changing cell membranes. Examples of antitumor antibiotics include dactinomycin, daunorubicin, doxorubicin (Adriamycin), idarubicin, and mitoxantrone. 4) Mitotic inhibitors. These drugs inhibit cell division or hinder certain enzymes necessary in the cell reproduction process. Examples of mitotic inhibitors include paclitaxel, docetaxel, etoposide (VP-16), vinblastine, vincristine, and vinorelbine. 5) Nitrosoureas.

These medications impede the enzymes that help repair DNA. Examples of nitrosoureas include carmustine (BCNU) and lomustine (CCNU) (American Cancer Society, 2001).

Previous studies have demonstrated that a wide range of anticancer agents, including chemotherapeutic agents, hormones, and various biologicals, induce apoptosis in malignant cells *in vitro* (Mesner *et al*, 1997; Kaufmann and Earnshaw, 2000). It is important to emphasize that this treatment-induced apoptosis is not merely a tissue culture phenomenon. Serial examination of peripheral blood mononuclear cells from acute leukemia patients undergoing induction therapy has demonstrated that various agents, including cytarabine, mitoxantrone, etoposide, paclitaxel, and topotecan, cause a marked increase in the number of apoptotic blasts (Li *et al*, 1994). Characteristic apoptotic changes have also been described in solid tumors after treatment of mice with various cytotoxic drugs, including cytarabine, 5-fluorouracil (5FU), fludarabine, doxorubicin, cyclophosphamide, cisplatin, etoposide, dactinomycin, and camptothecin (Kaufmann and Earnshaw, 2000).

For chemotherapy, natural products have been important sources of medicines for many traditional communities around the world. Natural products or their structural relatives comprise about 50% of the drugs that are used in cancer chemotherapy (Mann, 2002). In Malaysia, out of 12,000 species of higher plants which are found in this country, that are more than 1,000 species are said to have therapeutic properties and currently being used in the local traditional medicine system (Said, 1995). According to another report by Burkill in 1966, there are about 6,000-7,000 species of higher plants that have been

reported to have therapeutic or medicinal properties in Peninsular Malaysia and its surrounding islands (Burkill, 1966). Since the used of natural products in cancer chemotherapy have growth tremendously, the study of mechanism and mode of action of plant extracts become more and more important. In many research, plant related derivatives can be synthesized by knowing their biochemical reaction against cancer, and these derivatives can even have greater effect over the original compounds.

In this study, two natural compounds, 2',3'-epoxyisocapnolactone and 8-hydroxyisocapnolactone-2',3'-diol, which were isolated from the leaves of *Micromelum minutum*, were tested for their cytotoxic activity against human T-lymphoblastic leukemia cells (CEMSS)

The objectives of this study were:

- To evaluate the cytotoxic and antiproliferative activities of 2',3'-epoxyisocapnolactone and 8-hydroxyisocapnolactone-2',3'-diol against human T-lymphoblastic leukemic cells (CEM-SS) in terms of proliferation, morphological changes and the mode of cell death.
- To investigate the induction of apoptosis by 2',3'-epoxyisocapnolactone and 8-hydroxyisocapnolactone-2',3'-diol in treated CEM-SS cells.

CHAPTER 2

LITERATURE REVIEW

2.1 Cancer

Cancer, a general term for more than 100 diseases which characterized by an uncontrolled, abnormal growth of cells appear in different parts of the body that can spread to other parts of the body (Altman and Sarg, 1992). It is a potentially fatal disease caused mainly by environmental factors that mutate genes encoding critical cell-regulation proteins. The resultant aberrant cell behavior leads to expensive masses of abnormal cells that destroy surrounding normal tissue and can spread to vital organs resulting in disseminated, commonly a harbinger of imminent patient death (Alison, 2002). Cancer cells contain many genetic alterations that accumulate as tumors develop. Over the last 20 years, considerable information has been gathered on regulation of cell growth and proliferation leading to the identification of the involvement of specific genes at the molecular level (Macdonald and Ford, 1997).

The incident of cancer is rising with doubling in new cancer cases and cancers related deaths expected over the next 20 years (Alison, 2002). Part of the reason for this rise is that life expectancy is steadily rising and most cancers are more common in an ageing population. More significantly, a globalization of unhealthy lifestyles, particularly